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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Yamamoto, *et al.*	**Examiner:**	PAGONAKIS, Anna
Serial No.:	10/797,903	**Group Art Unit:**	1609
Filing Date:	March 10, 2004		
Title:	C-KIT KINASE INHIBITOR		

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

DECLARATION

I, Yuji Yamamoto, declare as follows:

1. I am a scientist at Eisai Co., Ltd. which is the Assignee of the present application.

2. My researches are at Eisai Co., Ltd. are in the area of cancer.

3. I received my master's degree in 1996 from Graduate School of Pharmaceutical Sciences, Tohoku University.

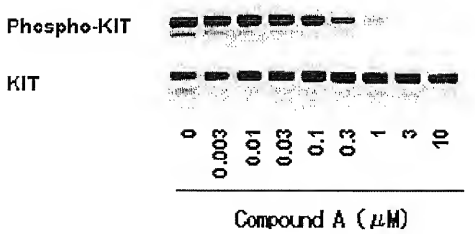
4. I am a co-inventor of the present application.

5. I performed Experiments A and B that are described on the following pages.

EXPERIMENT A

GIST882 cells were cultured in a 5% CO_2 incubator (37 °C) using an RPMI1640 medium (Sigma) containing 10% FBS (Cell Culture Technologies). Cells were harvested to prepare a cell suspension at a concentration of 5.0×10^5 cells/ml. 1 ml of this cell suspension was inoculated into each well of a 12-well plate and cultured in a 5% CO_2 incubator (37 °C) for 3 days. Then, 1 ml of FBS-RPMI1640 containing diluted Compound A (4-(3-Chloro-4-(cyclopropylaminocarbonyl)aminophenoxy)-7-methoxy-6-quinolinecarboxamide) was added to each well. After culturing in a 5% CO_2 incubator (37 °C) for 1 hour, the cells were washed with PBS, and SDS sample loading buffer was added and heated at 94 °C for 10 minutes and stored at -40 °C.

Part of the cell lysate sample was then electrophoresed on a 4-20% gradient polyacrylamide gel (Daiichi Pure Chemicals Co., Ltd.). After electrophoresis, lysate protein was transferred to a PVDF membrane (Amersham Pharmacia Biotech Inc.) for 3 hours. The transferred membrane was subjected to immunoblot using an anti-phospho-c-kit (Tyr719) antibody (Cell Signaling Technology Inc.) as a primary antibody and an anti-rabbit IgG, HRP-linked antibody (Cell Signaling Technology Inc.) as a secondary antibody. After the membrane was washed, it was developed with a Super Signal (Pierce Biotechnology, Inc.).



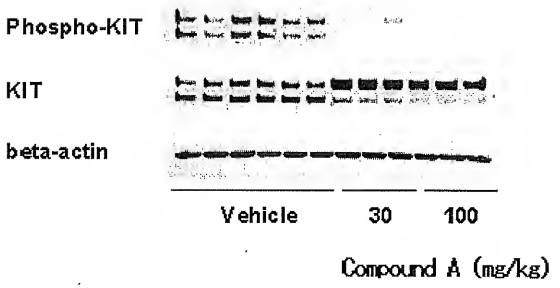
As shown in the above figure, Compound A inhibited the c-Kit hosphorylation in GIST cells in a concentration-dependent manner.

EXPERIMENT B

GIST882 cells were cultured in a 5% CO_2 incubator (37 °C) using an RPMI1640 medium containing 10% FBS, and harvested by conventional method using trypsin-EDTA. The cells were suspended in a mixture of phosphate buffer and Matrigel (1:1) to prepare a cell suspension at a concentration of 5.0×10^7 cells/ml. This cell suspension (0.2 ml) was transplanted subcutaneously into the flank region of nude mice (purchased from Charles River Laboratories, Inc.).

After transplantation, oral administration of Compound A (30 or 100 mg/kg; suspended in a 0.5% methylcellulose solution) was started at the point the tumor volume reached approximately 400-800 mm^3. Two hours after the oral administration, the tumor was resected, and a cell lysate buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mm $MgCl_2$, 1 mM EDTA (pH 8.0), 100 mM NaF, 1 mM PMSF, 10 μg/ml aprotinin, 50 μg/ml leupeptin, 1 μg/ml Peptatin A, 1 mM Na_3VO_4, 25 mM ß-glycerophosphate, and phosphatase inhibitor cocktail II (Sigma)) was added to the resected tumor, and the tumor was homogenized. After centrifugation, protein in the supernatant quantified, and a 3xSDS sample loading buffer was added to prepare a cell lysate sample. The cell lysate was heated at 94 °C for 10 minutes and stored at –40 °C.

The cell lysate sample was electrophoresed on a 4-20% gradient polyacrylamide gel (Daiichi Pure Chemicals Co., Ltd.). After electrophoresis, lysate protein was transferred to a PVDF membrane (Amersham Pharmacia Biotech Inc.) for 3 hours. Immunoblot was performed using an anti-phospho-c-kit (Tyr719) antibody (Cell Signaling Technologies, Inc.) or an anti-kit antibody (Santa Cruz) as a primary antibody and an anti-rabbit IgG, HRP-linked antibody (Cell Signaling Technologies, Inc.) as a secondary antibody. After the membrane was washed, it was developed with a Super Signal (Pierce Biotechnology, Inc.).



As shown in the above figure, Compound A inhibited c-KIT phosphorylation in implanted GIST tumor when administered at 30 or 100 mg/kg.

6. I, Yuji Yamamoto, declare that all statements made herein of my own knowledge are true and that these statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like are made punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the application or any patents that may issue thereon.

Respectfully Submitted,

Yuji Yamamoto

Yuji Yamamoto

Date: 2008/3/28